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(57) Abstract

The invention provides a peptide with lytic activity, having an amphipathic α-helix of sufficient length and character to allow the peptide to function lytically, wherein the N-terminal and/or C-terminal of said peptide comprises one or more moieties which result in an increased positive charge compared to the charge of a peptide of identical amino acid sequence and structure but not comprising said moiety. Methods of activation to provide activity and for inactivation of lytic activity, pharmaceutical compositions, and methods of treatment are described.

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LYTIC PEPTIDES

This invention relates to unique peptides having lytic activity, the lytic ability of which may be activated or inactivated.

These peptides are designed to be able to form amphipathic helixes. They may either be based on known sequences of natural products which form these structures, or may be designed from first principles using theoretical predictions of the sequences required to form such structures. The lytic peptides of the invention may be targeted to specific cells, for example by linking to a targeting moiety such as an antibody.

BACKGROUND OF THE INVENTION

In general, throughout this specification the standard three letter and single letter codes for amino acids are used. The terms "amino acid" and "residue" are used herein as synonyms.

Many living organisms are able to produce and secrete toxic compounds. Of these, snake, spider and insect venoms are perhaps the best known. One important class of naturally-produced toxic compounds is the cytotoxic amphipathic peptides. These include insect venoms such as melittin from bee venom, the magainins from frog skin, cecropins, bombolitins, mastoparans and related peptides, and the δ-haemolysin from *Staphylococcus aureus*. The cytopathic amphipathic peptides are generally basic, but vary widely in size and function. Melittin is 26 amino acids long, and is strongly cytotoxic and haemolytic; magainins are 23 amino acids long, are poorly haemolytic, and are toxic to bacteria. The bombolitins and mastoparans are only 14 amino acids long; they are cytotoxic, but have only weak haemolytic activity.

The cytotoxic amphipathic peptides have been extensively studied, particularly melittin and th

magainins, and many of the structural requirements for cytolytic function are now understood. For example, a melittin analogue with an ideal amphipathic α-helical sequence from positions 1-19, followed by the natural sequence from positions 20-26, has been synthesised; this polypeptide was more active than naturally-occurring melittin, showing that the haemolytic activity of this protein does not require the kink in the α-helix (DeGrado *et al*, J. Am. Chem. Soc., 1981 103 679-681; Dempsey *et al*, FEBS Letters, 1991 281 240-244). Melittin constructs with the reverse sequence (16-26)(1-13) retain their antibacterial activity, but show no haemolytic activity (Boman *et al*, FEBS Letters, 1989 259 103-106). Many other analogues of melittin have been synthesised, and it is suggested that some residues are essential, such as Lys_7 and Trp_{19}, and that the principal requirement for lytic activity is a large α-helical amphipathicity (Blondelle and Haughten, Biochemistry, 1991 30 4671-4678; Peptide Res., 1991 4 12-18; Blondelle *et al*, Biochim. Biophys. Acta., 1993 1202 331-336); a hydrophobic: hydrophilic ratio of at least 2:1 and a strongly positively-charged C-terminal region appears also to be essential (DeGrado *et al*, Biophys. J. 1982 37 329-338).

WO96/18104 by Torvey Pines Institute for Molecular Studies (Blondell *et al*) discloses methods for synthesis and screening of large numbers of melittin analogues having anti-microbial, haemolytic or catalytic achorly. The entire disclosure of WO96/18104 is incorporated herein by this cross-reference. This document discloses monomeric analogues of melittin, in which lys, is preferably replaced by glu or asp, more preferably glu.

More recently we have found that arginine residues are essential for the biological activity of melittin, while lysine is minimally important, in particular, Lys_7 can be replaced by serine without significantly affecting activity, and thus appears only to

be important in maintaining the amphipathic helix (Werkmeister *et al*, Biochem. Biophys. Acta., 1993 1157 50-64).

In addition to studies on characteristics of naturally-occurring lytic peptides, synthetic, highly amphipathic α-helices consisting only of Leu and Lys residues in a 2:1 ratio have been synthesised. Ranging from 12-22 residues long, these peptides were mainly α-helical, and were highly haemolytic, with activity varying according to the peptide length. The 15-, 20-, and 22-residue long peptides were 5 to 10 times more active than melittin, and more active than magainin or mastoparan by several orders of magnitude (Cornut *et al*, FEBS Letters, 1994 349 29-33).

The cytotoxic amphipathic peptides, particularly melittin, have been a target for intensive study because of their potential use as immunotoxins, when linked to antibodies as targeting agents (Dunn *et al*, Immunotechnology 1996 2 229-240). However, hitherto such applications have been limited, because the peptides are toxic to normal cells as well as to the target cells. In addition, in the case of melittin there is the risk of severe allergic side-effects. It appears that the characteristic cluster of Lys and Arg residues at the C-terminal, which is required for the activity of melittin (Schroder *et al*, Experientia, 1971 27 764-765); Habermann, in "Natural Toxins" D. Eaker and T. Waström, Pergamon Press, New York 1980 173-181) is also responsible for these allergic side-effects.

In work leading up to the present invention the inventors have found means of activating potentially lytic but inactive peptides. They have also found means of inactivating lytic peptides. These findings are important because they have implications for therapeutic uses of peptides whose lytic abilities can be activated and inactivated. Therapeutic uses are expected to include

cancer treatment, tr atment of infections and other conditions wh re it is desired to kill cells. The peptides of the invention may be used to form immunotoxins or the like, or as antibiotics. The peptides may also be used as biosensors.

The inventors have surprisingly discovered that peptides comprising an amphipathic α-helix having no lytic activity (or reduced lytic activity) may be activated by adding a group which neutralises the negative charge of the carboxy terminal residue. They have also found that by adding a positively charged amino acid to the amino terminal of an amphipathic α-helix this contributes to increasing lytic activity. Without wishing to be bound by theory it appears that the negative charge on the terminal residue in a number of peptides produced by the inventors is responsible for these proteins lacking lytic activity or having reduced lytic activity. It appears that neutralisation of the negative charge activates the inactive peptide. Thus removal of negative charge may act to cause an overall change in the net charge of the peptide and thereby render it lytically active. Alternatively, removal of negative charge may just act locally at the carboxy terminus rendering the peptide lytically active.

The inventors have also surprisingly found that the presence of an amino acid sequence or other group at the amino terminal of an otherwise lytically active peptide comprising an amphipathic α-helix, renders the peptide lytically inactive when the amino acid sequence or other group decreases the positive charge of the peptide. Removal of this amino acid sequence or group renders the peptide lytically active. Alternatively, and even more surprisingly, the inventors have found that such an inactive peptide can be activated by dimerisation.

It will be appreciated that various aspects of the invention are applicable to conjugate delivery systems, including immunotoxins, to antibacterial agents, and to

biosensor systems.

SUMMARY OF THE INVENTION

In a first aspect the invention provides a peptide with lytic activity, having an amphipathic α-helix of sufficient length and character to allow the peptide to function lytically, wherein the N-terminal and/or C-terminal of said peptide comprises one or more moieties which result in an increased positive charge compared to the charge of a peptide of identical amino acid sequence and structure but not comprising said moiety.

The peptide may be naturally occurring or non-naturally occurring.

The term "non-naturally occurring peptide" refers to a peptide in its entirety which does not occur in nature. Notwithstanding this the peptide may comprise fragments or parts which are based on natural sequences.

The term "amphipathic α-helix of sufficient length and character to allow the peptide to function lytically" means a peptide with a comparable function to that of the amphipathic α-helix of native melittin. This will generally be in the form of an α-helix which is about 20 amino acids in length. Preferably the peptides including the α-helix are between 21 and 26 residues in length.

The α-helix may be derived from naturally-occurring cytopathic amphipathic peptides, including melittin and other insect venoms, such as wasp, ant, or scorpion venom, antibacterial peptides such as a magainin, a cecropin, a bombolitin, or a mastoparan, or another known cytopathic amphipathic peptide. In the case of dimers they may be composed of α-helices which are the same or different. One or both of the amphipathic peptides may comprise a naturally-occurring sequence as described above, or portion thereof, together with an artificial sequence, as d scribed herein.

Ideally amphipathic α-helical sequences with positively charged residues periodically located along the peptide chain are known, and in some cases are several-fold more active than melittin. Fragments having this type of sequence may be used in the invention, either alone or as extensions of naturally occurring sequences.

The amino acids which make up the peptide of the invention may be naturally occurring amino acids, or non-naturally occurring analogues or homologues thereof, for example phenylglycine, norleucine or homoarginine. Such amino acids will be well known to those skilled in the art, for example as listed in WO96/18104. In one particularly preferred embodiment where the peptide is 21 amino acids or less the α-helix has at least 45% amino acid homology to native melittin.

The inventors' initial results indicated that the following amino acids may be essential to the lytic function of the α-helix : Lys or Arg 7 and Pro 14. Preferred amino acids are Gly 1, Gly 3, Ala 4, Thr 10, Gly 12, Ser 18 & Trp 19 or Phe 19.

We have subsequently found that the rules originally proposed were far too rigid, and that there is in fact very considerable latitude for substitution of amino acids. We now believe that the following are preferable, if not essential:

Hydrophilic amino acids at positions 3, 7, 10, 11, 14 and 18, of which at least two, preferably including the amino acid at position 7, should be Lys and/or Arg; and

Hydrophobic amino acids at positions 1, 2, 4, 5, 6, 8, 9, 12, 13, 15, 16, 17, 19 and 20. Advantageously the amino acid at position 14 is Pro or hydroxyproline.

The moieties which result in an increased positive charge may be amino acids or other chemical groups which are positively charged themselves, or which block negative charges which would otherwise occur on the peptide if the moiety was not present. The increase in the positive

charge may be an increase in the overall positive charge of the peptide, or a localised increase in positive charge. The latter may also have the effect of activating or increasing lytic activity.

Preferably the peptide of the invention is in the form of an isolated preparation which has been purified, to at least some degree.

In an alternative aspect related to the first aspect, the invention provides a non-naturally occurring peptide with lytic activity, having an amphipathic α-helix of sufficient length and character to allow the peptide to function lytically, wherein the C-terminal residue of said peptide is not negatively charged and wherein absence of a negative charge at the C-terminal residue is provided by blocking the negative charge of said residue.

The term "not negatively charged" means that the amino acid has a positive charge or is neutral.

Blocking the negative charge of the C-terminal residue may be achieved by any convenient means such as by addition of a neutral or positive substituent. Any convenient group which does not have a negative charge may be employed. Suitable substituents include $-NH_2$, acetamidomethyl (ACM) and the like. Optionally the C-terminal residue is a cysteine residue which has been neutralised. For example the cysteine residue may be substituted with ACM.

In a preferred embodiment of the first aspect, the invention provides a lytic peptide, comprising two amphipathic α-helices which are not themselves lytic, linked to form a dimer which has lytic activity.

The dimer may be a homodimer or a heterodimer. The nature of the link between the two components of the dimer is not critical; one convenient linkage is via a disulphide bond, which may be effected by having a cysteine or cysteine-amide residue at the C-terminal of each of the components of the dimer, or via a bridge moiety such as

$-CH_2S(CH_2)_nSCH_2-$, where n is 1 to 4. The only requirement is that the link moiety be able to hold the two peptide chains together. Preferably the link is of approximately the same size as a disulphide bond, but the person skilled in the art will recognise that many other linkages are possible. For example, maleic anhydride or maleic anhydride-like moieties such as those described in Australian Patent Application No. 90098/91, lysine, short peptides, or lysine-(aminocaproic acid)$_2$-lysine links may be used. The skilled person will readily be able to identify linkage groups which may be suitable, and to test whether in fact they are able to produce the desired effect.

The disulphide link is particularly convenient, as it can be directly expressed, using recombinant technology, or synthesised, and then simply oxidised under appropriate conditions, which are very well known in the art, to form the link. Optionally, there may be a bridging unit of CH_2 groups between the end of the peptide chain and the thiol group which is to be oxidised. A length of CH_2 chain which will be functionally active may readily be identified by routine testing

As described earlier, ideally amphipathic α-helical sequences with positively charged residues periodically located along the peptide chain are known, and in some cases are several-fold more active than melittin. Fragments having this type of sequence may be used in the invention, either alone or as extensions of naturally occurring sequences.

In addition to the wholly or partly naturally-derived sequences referred to above, an alternative embodiment of the first aspect of the invention provides a cytotoxic dimer of an amphipathic peptide, in which the amphipathic peptide has the general formula:

Ω Ω X X Ω Ω X Ω Ω X X Ω Ω (X, P or hypro) Ω Ω Ω X Ω Ω (C or Z)
or (C or Z) Ω Ω X X Ω Ω X Ω Ω X X Ω Ω P Ω Ω Ω X Ω Ω;

more preferably

Ω Ω Σ Σ Ω Ω (K or R) Ω Ω Σ Σ Ω Ω P Ω Ω Ω Σ Φ Ω (C or Z)
or Ω Ω Ω Ω Ω Ω (K or R) Ω Ω Σ Σ Ω Ω P Ω Ω Ω Σ Φ Ω (C or Z)
or (C or Z) Ω Ω Σ Σ Ω Ω (K or R) Ω Ω Σ Σ Ω Ω P Ω Ω Ω Σ Φ Ω
or Ω Ω Ω Ω Ω Ω (K or R) Ω Ω Σ Σ Ω Ω P Ω Ω Ω Σ Φ Ω

and wherein the symbols have the following meanings:

Ω = glycine; a hydrophobic amino acid including but not limited to alanine, isoleucine, leucine, valine, and norleucine; or ϕ;

Σ = asparagine, glutamine, serine or threonine;

Φ = phenylanine, tryptophan, tyrosine, a non-naturally occurring aromatic amino acid, or a synthetic aromatic residue; and

X represents hydrophilic amino acids, at least one of which is a basic amino acid such as lysine, arginine or homoarginine; preferably two or more of the hydrophilic amino acids are basic amino acids.

R = is arginine

K = lysine

hypro = hydroxyproline

P = proline

C = cysteine

Z = is an amino acid which is not negatively charged, or is either a cross-linking agent, such as a commercially available heterobifunctional linking group, or a blocking group; when Z is a blocking group it may be $-NH_2$, ACM or the like.

C or Z may be at either end of the peptide, but not at both ends.

Preferred sequences are:

G I G A V L K V L T T G L P A L I S W I C -NH_2 (Peptide 01B)
C G I G A V K L V L T T G L P A L I S W I -NH_2 (Peptide 29)
2(G I G A V L K V L T T G L P A L I S W)K -NH_2 (Peptide 30)
X L Q S L V S L V Q S L V S L V L W F L R S R K N N -NH_2 (Peptide 34)
L L Q S L V K L V Q S L V P L V L Q F L C -NH_2 (Peptide 42)
L L Q S L V K L V Q S L V S L V L Q F L C -NH_2 (Peptide 44)
G L G A L V K L V T S G V P L V L S W L C -NH_2 (Peptide 56)
A V R G I G A V L K V L T T G L P A L I S W I C -NH_2 (Peptide 50)
Ac A V R G I G A V L K V L T T G L P A L I S W I C -NH_2 (Peptide 50A)
G I G A V L K V L T T G L P A L I S W I M-NH_2 (Peptide 64M)
S S L G I K A V L K V L T T G L P A L I S W I A-NH_2 (Peptide AP11)
S S L G I G A V L K V L T T G L P A L I S W I A-NH_2 (Peptide AP13)

where -NH_2 indicates the C-terminal amide group.

It appears that an aromatic residue designated Φ is beneficial for function. Preferably this is at residue 19, as in Peptide 01B. Either a synthetic aromatic residue or a non-naturally occurring aromatic amino acid may be used as alternatives to naturally occurring aromatic amino acids. See for example U.S. Patent No. 5294605 by Scripps Research Institute.

The cross-link which forms the dimer may be at either the C-terminal or the N-terminal end of the peptide. While terminal cysteine or lysine residues are particularly convenient for formation of the cross-link, as discussed above the skilled person will be aware of alternative means of linking the two peptide chains to form a dimer. For example, a cross-link at the C-terminal end, as in peptide 01B, can be achieved using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in conjunction with a suitable diamine, such as tetramethylenediamine (putrescine). An alternative cross-link at the N-terminal can be effected using a reagent such as 2,3-dibromopropionyl-N-hydroxysuccinimide ester (McKenzie *et al*, J. Protein Chem., 1988 7 581-592).

The length of the bridge between the two peptide chains is relatively flexible, and the disulphide can be replaced by a -CH_2 S $(CH_2)_n$S CH_2-crosslink (where n is 1-4),

by a lysine, by small lengths of peptide, or by other suitable chemical moieties.

These peptides may be amidated, and/or acetylated at the N-terminal.

A typical example is based on melittin, which has the following sequence:

G I G A V L K V L T T G L P A L I S W I K R K R Q Q -NH_2
1 10 20 26

The potent lytic activity of melittin is well established (Habermann and Jentsch 1967), as is the loss of activity on removal of residues 21-26 (Schroder *et al* 1971).

A typical peptide is synthesised containing the first 20 residues of the melittin sequence, with the addition of a cysteine-amide residue as residue 21 in the sequence. This peptide is oxidised to form the dimer as a disulphide. Potentially any sequence which forms an amphipathic helix could be used, provided that certain key residues are present.

In a particularly preferred embodiment of the invention, the helical dimer is

G I G A V L K V L T T G L P A L I S W I C
|
G I G A V L K V L T T G L P A L I S W I C

The peptides of the invention may be synthesised, using conventional methods such as solid phase synthesis, particularly if a C-terminal amide such as a cysteine amide is desired. Where the cross-link is via lysine, the dimeric peptide can be directly synthesised by forming peptide bonds at both the α-NH_2 and the ε-NH_2 groups of lysine, ie. lysine can form a branch point. Alternatively, they may be expressed using r combinant DNA technology;

this is of course not suitable for C-terminal amides, but this modification could be introduced subsequently. The means of production may be selected depending on the use for which the peptide is intended.

The peptide of the invention may be attached to a carrier molecule, either by chemical linking or by synthesis as a fusion protein using recombinant methods. The monomeric form of a peptide of the invention may be expressed in a host cell, for example as a fusion protein with a fusion protein carrier, and then either the peptide-carrier hybrid may be dimerised, or if the peptide is to be in the form of a heterodimer, a suitable second half of the dimer may be added either by chemical linkage or by expression as a fusion protein to complete the dimeric peptide of the invention. For example, if the fusion protein carrier is an Fab fragment of an antibody, one peptide of the invention could be expressed fused to the heavy chain and the second peptide of the invention expressed fused to the light chain of the Fab.

In either synthetic method, the peptide may be linked to a cleavable affinity or detection label; several suitable labels are known in the art, for example FLAG™ (U.S. Patent No. 4,703,004), T7-Tag and HSV-Tag (Novagen), and BTag (our International Patent Application No. PCT/AU96/00516 entitled "Epitope Tagging System").

Preferably the carrier molecule is selected from the group consisting of an antibody, an Fab, Fv, ScFv or other antigen-specific fragment or analogue thereof, epidermal growth factor (EGF) and transforming growth factor-α (TGF-α). Where the antibody, analogue or fragment is or is derived from a monoclonal antibody it may be modified to reduce immunogenicity, eg. by "humanization". Suitable methods are well known in the art. Preferably the antibody is specific for a cell surface antigen and more preferably is specific for a cancer-specific antigen. In one embodim nt this antigen is specific for a solid tumour.

Many such antigens ar known in the art, such as carcinoembryonic antigen, which is associated with gastrointestinal and lung carcinomas, prostate carcinoma antigen, ovarian carcinoma antigen, and breast carcinoma antigen.

In an alternative embodiment related to the first aspect the invention provides a peptide which is capable of lytic activity, comprising an amphipathic α-helix of sufficient length and character to allow the peptide to function lytically, and

(a) a cleavable moiety located amino terminal to said α-helix, wherein said moiety prevents or retards lytic function of said peptide and wherein removal of said moiety results in a lytically active peptide, and/or

(b) a negatively-charged carboxy terminal residue of said peptide,

wherein the negative charge prevents or retards lytic function of said peptide and wherein removal of said charge by blocking with a suitable group results in lytic activity.

The cleavable moiety may be any moiety which can be linked to the α-helix and inactivates lytic function. Preferably the moiety is an amino acid sequence such as a pre-sequence Acetyl- AVY-, Acetyl-SSGYSNT- or Acetyl-AVE-. Preferably the cleavable sequence is selectively removable by enzymatic means.

Suitable groups for blocking the negative charge may be any group without a negative charge which does not otherwise adversely affect the lytic ability of the peptide. Suitable groups include other amphipathic α-helical peptides, particularly those able to form a dimer with the peptide capable of lytic activity. Other suitable groups include $-NH_2$ and ACM. While $-NH_2$ does not preclude dimer formation where the terminal residue is cysteine, ACM, which binds the thiol group prevents dimer formation.

In a third aspect the present invention provides

a method of modulating the lytic activity of a peptide, said peptide comprising an amphipathic α-helix of sufficient length and character to allow the peptide to function lytically, and optionally comprising additional amino acids, said method comprising, under suitable conditions,

(a) increasing the positive charge of said peptide by adding to the N and/or C terminal one or more moieties which increase the positive charge of the peptide, or

(b) decreasing the positive charge of said peptide by removing from the N and/or C terminal one or more moieties which decrease the positive charge of the peptide

wherein step (a) results in an increase of lytic activity or activation of lytic activity and step (b) results in a decrease or inactivation of lytic activity.

The term "modulating" means changing or altering the lytic ability of the peptide and includes changing a lytically inactive peptide into a lytically active peptide and the reverse.

The moieties which increase or decrease the positive charge may be those described earlier.

The term "adding to the N and/or C terminal" refers to chemically linking the moiety to one of the available groups on the terminal residue. The term "removing from the N and/or C terminal" refers to cleaving or otherwise chemically removing the moiety in question from the remainder of the peptide.

The term "under suitable conditions" means carrying out the method in the presence of the appropriate chemicals, buffers, temperature, for an appropriate time. Such conditions will be known by those skilled in the art.

In an alternative embodiment related to the third aspect the invention provides a method of activating a lytically inactive peptide, wherein said inactive peptide

compris s an amphipathic α-helix of sufficient length and character to allow the peptide to function lytically and

(a) a cleavable moiety located amino terminal to said α-helix, wherein said moiety inactivates lytic function of said peptide and wherein removal of said moiety results in a lytically active peptide, and/or

(b) a negatively charged carboxy terminal residue wherein the negative charge may be removed by blocking with a suitable group which results in lytic activity

said method comprising, under suitable conditions, treating said peptide with

(c) a cleaving agent to remove said moiety and/or

(d) a blocking agent to remove the negative charge of said residue

such that said peptide becomes lytically active.

The cleaving agent may be any suitable agent to remove the cleavable moiety. Where the cleavable moiety is an amino acid sequence the cleaving agent may be an appropriate enzyme.

The blocking agent may be any agent suitable for blocking the negative charge. In the case where the peptide comprises a cysteine residue, for example, the blocking agent may be another amphipathic α-helical peptide which contains a cysteine residue and is capable of forming a dimer. Other suitable blocking agents may be the suitable groups described above.

The term "under suitable conditions" in relation to the cleaving agent and/or blocking agent means treating the peptide under appropriate reaction conditions and for an appropriate time such that the cleaving agent and/or blocking agent can perform their desired functions. Such conditions will be determined by the nature of the peptides and the agents used and will be well known to those skilled in the art.

In another alternative embodiment related to the third aspect the invention provides a method of

inactivating a lytically active peptid wherein said peptide comprises an amphipathic α-helix of suffici nt length and character to allow the peptide to function lytically, and/or a non-negatively charged carboxy terminal residue, and where said peptide is optionally in the form of a dimer comprising two α-helices, said method comprising, under suitable conditions,

(a) linking a cleavable moiety amino terminal to said α-helix and/or

(b) creating a negative charge on said carboxy terminus.

Preferably the cleavable moiety is an amino acid presequence which reduces the overall positive charge of the peptide.

According to a fourth aspect the invention provides a pharmaceutical composition, comprising a peptide of the invention, either alone or coupled to a carrier molecule, together with a pharmaceutically-acceptable carrier. Optionally the composition may also comprise an antibiotic or a toxic anion, such as fluoride ion, or an anti-tumour, antibacterial, anti-viral or antiparasite agent.

Melittin is known to be able to inhibit the replication of human immunodeficiency virus (International Patent Publication No. WO 91/08753), and it is contemplated that the peptides of the invention will be useful for this purpose, as well as in the treatment of cancer.

According to a fifth embodiment, the invention provides a method of treatment or alleviation of cancer or of a manifestation of infection with human immunodeficiency virus, comprising the step of administering an effective amount of a peptide of the invention, either alone or coupled to a carrier molecule, to a subject in need of such treatment.

According to a sixth embodiment, the invention provides a method of treatment of a microbial infection,

comprising the step of administering an effective amount of a peptid of the invention to a subject in need of such treatment, said dimer comprising an amphipathic helical sequence of the invention and a cecropin or a cecropin-like peptide.

According to an seventh embodiment, the invention provides a biosensor, comprising a first monomeric form of a peptide of the invention linked to an antigen and a second monomeric form of a peptide of the invention linked or adsorbed to a solid support or to a membrane, a receptor, or a bound antibody or Fab, Fv, scFv or other antigen-binding fragment or analogue thereof, such that an analyte to be detected induces dimerisation of the monomers.

Dimerisation may be detected by membrane lysis or by ion-channel formation. In the latter case, the dimeric form of the molecule constitutes an voltage-gated ion channel, so that dimerisation results in current flow. Preferably dimerisation is effected via formation of a disulphide bond, so that the biosensor of this aspect of the invention provides a redox switch.

Detailed Description of the Invention

The invention will now be described in detail by way of reference only to the following non-limiting examples, and to the figures, in which:

Figures 1 to 6 show flow cytometric analysis of the effect of cytolytic peptides on CEM T-cell lymphoma cells. The cells were treated with the peptides for 10-30 minutes and analysed by light scattering and propidium iodide penetration, indicated by fluorescence.

Figure 1A shows flow cytometric analysis of untreated control cells, with side scatter (vertical axis) plotted against forward scatter (horizontal axis),

Figure 1B shows the viability of control cells,

as measur d using exclusi n of propidium iodide; cell count (vertical axis) is plotted against propidium iodide fluorescence (horizontal axis);

Figure 2 shows the effect of intact melittin on cells;

Figure 3 shows the effect of peptide 29;

Figure 4 shows the effects of peptide 01B;

Figure 5 shows the effects of peptide 10;

Figure 6 shows the effects of peptide 34;

In each of Figures 2 to 6, Figure A shows flow cytometric analysis, Figure B shows cell viability;

Figure 7 shows the effects of peptide AP11;

Figure 8 shows the effect of melittin on cell membrane potential, as measured by formation of ion channels, indicated using the dye $DiSc_3$. Figure 8A shows control cells, Figure 8B shows melittin-treated cells.

Figure 9 shows diagrammatic representations of immunotoxin molecules constructed according to the invention.

Figure 9a depicts an immunotoxin in which the inactivating leader peptide is joined by a proteolytic cleavage sequence (X) to a cytolytic peptide joined to a scFv shown here as VH-VL. One example of the strategy for the genetic construction of immunotoxin molecules is shown in figure 10.

Figure 9b depicts an immunotoxin in which the chemically or genetically conjugated Fab comprising one heavy chain fused to a proteolytically sensitive linker sequence (X) fused to a cytolytic peptide and a second light chain molecule fused to another proteolytically sensitive linker sequence (X) fused to another cytolytic peptide. The genetic conjugation is in the description of figure 10.

Figures 9c and 9d depict reverse orientation forms of 9a and 9b.

Figure 10 shows the specific oligonucleotid s used to construct an scFv expression cass tte for the NcoI-EcoRI restriction sites of pGC. The PCR cloning strategy is also shown using these oligonucleotides. The genetic conjugation of scFv or Fab to cytolytic peptide and proteolytic cleavage signal, and construction of expression cassettes use PCR and cloning techniques that are published (Coia G., Hudson, P.J. and Lilley G.G. (1996) Journal of Immunological Methods. 192, 13-23).

Following our study of lytic peptides suitable for the construction of immunotoxins (Werkmeister *et al*, Biochim. Biophys. Acta., 1993 1157 50-54), we synthesised a peptide consisting of the first 20 residues of melittin with the addition of a C-terminal cysteine amide. The intention was to couple this peptide to a monoclonal antibody (Mab) through the cysteine thiol group.

Published reports suggest that the 21 residue peptide could not be lytic in its own right, but might assume this activity on delivery to the cell membrane by an Mab (Schroder *et al*, *op.cit.*). Surprisingly, the peptide was found to have high lytic activity, which we have now found was the result of the formation of a dimer, formed from the oxidation of the cysteine thiol.

Native melittin, free of phospholipase, was purchased from Fluka Chemie AG, Switzerland and used without further treatment. Synthetic melittin was obtained from Auspep, Australia.

Example 1 Synthesis of Peptides

Native melittin, free of phospholipase, was purchased from Fluka Chemie AG, Switzerland and used without further treatment. Synthetic melittin was obtained from Auspep (Australia).

Synthetic amphipathic peptides were designed according to the principles set out in the review by Kaiser and K zdy (Proc. Natl. Acad. Sci. USA., 1983 80 1137-1143).

Ac represents acetyl and X represents norleucine. Norleucine was inserted into peptides 10 and 34.

The peptide which we have designated OIB, of sequence

H-G I G A V L K V L T T G L P A L I S W I C -NH_2

was synthesised on an Applied Biosystems 430A Peptide Synthesizer, using the FastMoc strategy. RINK resin (Rink: Tetrahedron Letters, 1987 728 3787) was used as the substrate for the assembly of this peptide, yielding the peptide as the amide on cleavage with trifluoroacetic acid. The cysteine side chain was protected by trityl, serine and threonine by t-butyl and lysine by t-butyloxycarbonyl groups. The peptide was purified by reverse-phase HPLC using a VYDAC C18 column. Separation was achieved by using a 20 minute gradient from 5 to 60% acetonitrite. The integrity of the peptides was established by amino acid analysis and electrospray mass spectrometry. The peptide oxidised when dissolved in dimethyl sulphoxide in the presence of air to yield the disulphide dimer. Monomeric peptide (01D) was prepared by alkylating with iodoacetic acid in dimethylformamide. Monomeric peptide 63 was prepared as described above using ACM.

The lysine branched dimer, peptide 30, was synthesised by the technique described above, but FmocLys(Fmoc)OH was used at the C-terminal. This yielded a crude product which consisted of a mixture of deletion peptides along with the desired peptide which was purified by C_{18} reverse phase HPLC. The monomeric cysteine peptides 01b and 42C were prepared by alkylating peptides 01B and 42 with iodoacetic acid in dimethylformamide, after prior reduction with tributylphosphine.

The acetylated peptides, 01C, 42B, 44B and 46B

were obtained by treating the parent peptides with an excess of acetic anhydrid in dimethylformamide containing a trace of triethylamine.

Other peptides, whose sequences are given below, were synthesised essentially as described above and likewise, where appropriate, dimerised by oxidation of the thiol of the cysteine residues.

G I G A V L K V L T T G L P A L I S W I C -NH_2	(Peptide 01B)
Ac-G I G A V L K V L T T G L P A L I S W I C -NH_2 (Ac on K)	(Peptide 01C)
G I G A V L K V L T T G L P A L I S W I C -(SCH_2COOH)-NH_2	(Peptide 01D)
Ac-X L Q A L L S L L Q S L L S L L L Q F L R R K R Q Q -NH_2	(Peptide 10)
C G I G A V L K V L T T G L P A L I S W I -NH_2	(Peptide 29)
2(G I G A V L K V L T T G L P A L I S W)K -NH_2	(Peptide 30)
X L Q S L V S L V Q S L V S L V L Q F L R S R K N N -NH_2	(Peptide 34)
G L G A L V K L V T S G V P L V L S W L C -NH2	(Peptide 56)
G I G A V L K V L T T G L P A L I S W I C -($SCH_2NHCOCH_3$) -NH_2	(Peptide 63)
L L Q S L V S L V Q S L V S L V L Q F L C -NH_2	(Peptide 41)
L L Q S L V K L V Q S L V P L V L Q F L C -NH_2	(Peptide 42)
Ac-L L Q S L V K L V Q S L V P L V L Q F L C -NH_2 (Ac on K)	(Peptide 42B)
L L Q S L V K L V Q S L V S L V L Q F L C -NH_2	(Peptide 44)
Ac-L L Q S L V K L V Q S L V S L V L Q F L C -NH_2 (Ac on K)	(Peptide 44B)
L L Q S L V R L V Q S L V P L V L Q F L C -NH_2	(Peptide 46)
L L Q S L V K L V Q S L V P L V L Q F L C(SCH_2COOH) -NH_2	(Peptide 42C)
Ac-L L Q S L V R L V Q S L V P L V L Q F L C -NH_2	(Peptide 46B)
A V R G I G A V L K V L T T G L P A L I S W I C -NH_2	(Peptide 50)
Ac-A V R G I G A V L K V L T T G L P A L I S W I C -NH_2	(Peptide 50A)
S S L G I K A V L K V L T T G L P A L I S W I A -NH_2	(Peptide AP11)
S S L G I G A V L K V L T T G L P A L I S W I A -NH_2	(Peptide AP 13)

In these peptides, phenylalanine can be substituted for tryptophan without loss of activity.

Example 2 Haemolytic Effects

The haemolytic effect of the peptides of Example 1 was compared with that of melittin.

Peptid s were dissolved in dimethyl sulphoxide (DMSO) at 5 mg/ml and serially titrated by two-fold dilutions in phosphate buffered saline (PBS). The final concentrations in the 96-well U-bottomed microtitre plates (Nunc, Denmark) ranged from 200µg/ml to 0.7µg/ml. 0.6% suspension of washed human red blood cells (100µl) were added for 1 hour. Plates were centrifuged at 150 X g for 5 min, and 100µl aliquots were transferred to a 96-well polyvinyl chloride plate (Dynatech Laboratories, Alexandria, VA). Haemolysis was assessed by measurement of optical density at 405nm with an automatic EAR 400 SF ELISA plate reader (SLT Lab instruments, Groedig/Salzburg, Austria). The percentage of haemolysis was calculated by comparison with absorbances from a buffer blank ("no lysis" control) and a sample treated with 0.1% Triton X-100 ("maximum lysis" control). Lytic activity could be calculated from the linear portion of the haemolytic titration curve for each peptide where one lytic unit was defined as the concentration of peptide required to produce a given percentage of haemolysis. The relative activities of peptides were assessed directly from the linear portion of the titration curves.

The results are summarised in Table 1.

Table 1

% Haemolysis Induced by Peptides, as compared to Melittin (100%)

Peptide	Final Concentration of Peptide (µg/ml)								
	200	100	50	25	12	6	3	1.5	0.7
Melittin	100	100	100	100	100	65	51	14	4
01B	100	100	96	58	29	14	9	4	2
01B	97	100	96	83	41	17	12	9	9
01C	1	0	1	0	0	0	0	0	0
01D	1	0	1	1	0	0	0	0	0
10	100	100	100	98	97	93	35	9	2
29	100	80	55	33	15	9	7	4	3
30	100	92	79	57	33	17	12	8	5
34	100	100	99	76	35	15	10	10	1
41	1	7	5	1	1	0	0	0	0
42	68	29	12	9	9	6	6	2	2
42 B	4	2	6	5	6	3	2	0	1
42 R/A	11	8	7	2	1	0	0	1	1
44	28	15	7	2	0	0	0	0	0
44 B	4	4	7	4	4	3	3	2	1
42	67	45	22	9	5	2	5	2	2
42B	7	5	5	5	4	5	5	4	3
42C	11	8	7	2	1	0	0	1	1
46	81	61	33	7	0	0	0	0	0
46B	2	0	2	1	1	1	0	0	0
50	99	95	100	86	71	36	18	4	0
50A	98	94	80	46	8	2	2	0	0
56	100	100	100	85	49	14	0	0	0
63	96	98	89	53	30	3	5	5	5
64M	100	100	91	91	88	88	54	48	13
AP11	100	97	81	34	10	5	3	3	4
AP13	92	93	89	38	8	2	1	1	1

Peptide 29 : C-terminal cysteine

Peptide 30 : dimer cross-linked via lysine residues

R/A : reduced and alkylated

Table 1 summarises the haemolyic effects of all peptid s after 1 hour at vari us concentrations ranging from 200 µg/ml to 0.7 µg/ml. Peptide 10 is based on the sequence published by Degrado et al ((1981) J.Amer.Chem.Soc.103:884-890; the variations being the replacement of the N-terminal leucine with N-acetyl norleucine, tryptophan 19 with phenylalanine and lysine 21 with arginine. Despite retaining only 31% sequence identity with melittin, peptide 10 has a similar lytic activity to melittin (Table 1). Likewise peptide 34, which has no sequence identity with melittin has only a slightly reduced activity, somewhere around a 4-fold reduction.

Peptide 01B, the truncated (C-terminus-deficient) analogue of melittin was found to be highly active causing significant haemolysis at concentrations as low as 12 µg/ml. The N-acetylated form of this peptide (peptide 01C) and the monomer (peptide 01D) were both completely inactive even at the highest concentrations of 200 µg/ml. Dimerisation of this truncated peptide either by an N-terminus cysteine (peptide 29) or by a C-terminus lysine (peptide 30) also resulted in highly lytic peptides, slightly less toxic than peptide 01B (Table 1).

Peptide 41, the truncated (C-terminus-deficient) analogue of the lytic peptide 34, was completely inactive even at the highest concentration. Substitution of lysine and proline into this sequence at positions 7 and 14 respectively (peptide 42), resulted in significant lytic activity, albeit still around 4-fold less than peptide 34 on a molar basis. Both substitutions were necessary since replacement with only lysine at position 7 (peptide 44) resulted in only a mild lytic activity. Replacement at position 7 with arginine (in association with substitution of proline at position 14) produced a highly lytic peptide (peptide 46, Table 1) which was equally if not more active than peptide 42. In all these studies with amino acid substitutions, the haemolytic activity of the resulting

peptides required a free unacetylated N-terminus (peptides 42C, 01C, 42B, 44B and 46B.

We also examined the activity of monomeric peptides whose sequence was based on that of melittin. Peptides 10 and 34, each of which has N-terminal norleucine, leucine replacing lysine$_7$, and serine replacing proline$_{14}$, both have haemolytic activity comparable to that of melittin. However, neither of these peptides is active in the dimeric form. Thus, contrary to the results of Blondell and Houghton (*op.cit.*) it appears that a residue corresponding to lysine$_7$, is not required for activity of the monomeric peptide; nonetheless, this residue confers maximum activity on the dimer, as does a proline corresponding to proline$_{14}$.

In Peptide 29, cross-linking is via a cysteine added at the C-terminal of the melittin sequence, so that the lysine is at position 8 and proline is at position 15 in the sequence of this peptide; good activity was observed, so it is clearly essential only that a lysine and a proline occupy these relative positions in the sequence.

Example 3 Measurement of Cytolytic Effect by Flow Cytometry

To assay the effects of cytolytic peptides on cells, we have utilised flow cytometry as described by Weston *et al* (Cytometry, 1994 15 141-147), with some modifications. Flow cytometry uses analysis of the scattering of laser light from cells moving in a fluid stream to give information about the state of the cells. Light scattered in the direction of the laser beam (forward scatter) is measure of cell size. Light scattered at right angles to the beam (side scatter) is an indication of granularity within the cell, and hence of cell membrane integrity. In addition, fluorescent molecules can be added to the cells, and their specific fluorescence when excited by the laser beam can be measured by light emission at right angles to the laser beam. This fluorescence can give

information about the composition or state of the cells, depending on the specific fluorescent dye used.

Weston *et al* showed that cells change their forward and side light scattering properties when acted upon by melittin. We have measured these changes, and have also incorporated two additional parameters. The first is cell viability measured by the exclusion of the dye propidium iodide. Cells with intact membranes do not take up this dye, and do not become fluorescently stained. Cells with broken membranes take up the dye, which binds to their internal nucleic acids and becomes brightly fluorescent. Cell viability can therefore be measured at the same time as light scattering, and is indicated by the proportion of cells that are not stained by propidium iodide. As an index of peptide cytolysis, forward light scatter was measured and plotted against cell membrane integrity which was assessed by exclusion of the dye propidium iodide (Shapiro (1994) Practical Flow Cytometry, Third Edition, Wiley Liss New York). CEM T cell lymphoma cells (250µl) were used for the cytolytic assay, at a concentration of 10^6 cells/ml in PBS. Cells were incubated at room temperature in the presence of peptides and 4µg/ml propidium iodide (Sigma Chemical Company). The concentration of peptide (100µg/ml) was chosen from the haemolytic titration curves. Flow cytometry was carried out on a Coulter EPICS® Elite flow cytometer with illumination at 488nm. Forward light scatter was measured at 488nm and propidium iodide fluorescence was detected at greater than 600nm.

Fluorimetric measurement of cell membrane potential was also determined. Some carbocyanine dyes dissolve in cell membranes and give a fluorescent signal which is dependent on the orientation of the dye molecules within the cell membranes. (Cohen & Salzberg (1978) Rev. Physiol. Pharmacol. 83:35-88). This orientation is dependent on th voltag difference across the cell

membrane and therefor th intensity of the flu rescent signal is pr portional to the voltage drop. Cells that are effected by cytolytic peptides develop ion channels that decrease the cell membrane potential, resulting in a rapid decrease in dye fluorescence. We have used the carbocyanine dye, $DiSC_3$ (Ash *et al* (1978) Biochim. Biophys. Acta 1030:1-10) [3,3'-diisopropylthiadicarbocyanine iodide] (Molecular Probes, Eugene, Oregon), to measure cell membrane potential and indicate the formation of ion channels after addition of peptides (100µg/ml) for 5 minutes. For membrane potential measurements cells, in PBS, were pre-loaded with 0.05µM $DiSc_3$ in normal saline for 15 min. at room temperature. Flow cytometry was carried out as above with illumination at 488nm for light scatter and at 633nm for excitation of $DiSC_3$ fluorescence. Fluorescence of the $DiSC_3$ was measured at greater than 633nm. Because the fluorescence emission spectra of propidium iodide and $DiSC_3$ overlap (Schapiro (1994) op cit), the two dyes could not be used simultaneously.

Results with untreated control cells are shown in Figure 1, and results with intact melittin are shown in Figure 2. Figures 3, 4, 5, 6 and 7 respectively show results with peptide 29, peptide 01B, peptide 10, peptide 34 and peptide AP11.

The dots on the diagram in A for each figure are measurements on individual cells, and their density indicates the number of cells in each area of the diagram. Figure 1A shows a normal cell population. It can be seen that the measurements of most cells lie in an area to the bottom centre of the diagram. Dead or damaged cells have measurements in the bottom to middle left of the diagram. A shift from the normal cell area toward the left or top of the figure is an indication of cell damage.

Figure 1B shows normal cells that are not fluorescent, indicated by the histogram peak against the left axis of the graph. Dead or damaged cells have

fluorescence that is greater than that observed for the normal c lls, indicated by a shift in the histogram toward the right of the diagram.

It is evident from the figures that the different peptides have differential effects on light scattering and cell viability.

Example 4 Effect of Cytolytic Peptides on Cell Membrane Potential

Carbocyanine dyes are fluorescent probes which dissolve in cell membranes and give a fluorescent signal which is dependent on the orientation of the dye molecules within the cell membranes. This orientation is dependent on the voltage difference across the cell membrane, and therefore the intensity of the fluorescent signal is proportional to the voltage drop. Cells that have been affected by cytolytic peptides develop ion channels that decrease the cell membrane potential, resulting in a shift of the fluorescence intensity histogram towards the left of the diagram. We have used the carbocyanine dye $DiSC_3$ (3,3'-diisopropylthiadicarbocyanine iodide; Molecular Probes, Eugene, Oregon) to measure cell membrane potential and indicate the formation of ion channels. This procedure is a very sensitive means of demonstrating the effects of cytolytic peptides. CEM T cell lymphoma cells were incubated with 5 mM $DISC_3$ in normal saline for 15 minutes at room temperature, and followed over a period of 0 to 10 minutes.

Results with untreated control cells and with cells treated with melittin for 5 min are shown in Figure 8A and Figure 8B respectively. It is evident that almost the entire population of cells is rendered permeable by melittin.

Example 5 Constructi n of Peptides Capable of Lytic Activity

Peptides capable of lytic activity in accordance with the second aspect of the invention may be produced in accordance with the following. While the following relates to dimers it will be appreciated that monomeric peptides may be similarly produced.

Synthesis: The peptide H - G I G A V L K V L T T G L P A L I S W I C -NH_2 was synthesised and oxidised to produce a disulphide dimer basically as described in Example 1.

Peptides such as 01B, 42 and L L Q S L V R L V Q S L V P L V L Q F L C -NH_2 (Peptide 46) may be synthesised as described in Example 1. The inactivating presequence may be added during peptide synthesis or the relevant nucleic acids encoding it may be included in the construct if the peptide is being made by recombinant means. It should be noted that phenylalanine may be substituted for tryptophan with only minimal loss of lytic activity.

The length of the bridge between the two peptide chains is relatively flexible and the disulphide can be replaced by either a - CH_2 S $(CH_2)_x$ S CH_2- (where x is 1-4) crosslink or a lysine or small lengths of peptide or other chemical moiety.

Deactivation:

We have discovered that the peptides described above, and related sequences, can be rendered inactive by the addition of an appropriate pre-sequence, then reactivated by treatment with an appropriate enzyme. The presequences are added as required and their sequence being dependent on the enzyme which is planned to cleave at the appropriate site.

Typical presequences: (inactivated by)

Acetyl-A V Y- (Prostate Specific Antigen or Chymotrypsin)

Acetyl- S S G Y S N T - (PSA or Chymotrypsin)

Ac tyl-A V E- (*Staphylococcus aureus* V8 protease, Houmard, J. & Drapeau, G. R. (1972), Proc.Natl.Acad.Sci.USA, 69,3506-3509)

Many other presequences are possible, only requiring a specific cleavage site which is not present in the main peptide chain.

Example 6 Construction of Additional Peptides

The following peptides are constructed in accordance with Example 1 and tested for their haemolytic activity.

G I G A V L K V L T T G L P A L I S W I A-NH_2	(Peptide 64A)
G I G A V L K V L T T G L P A L I S W I N-NH_2	(Peptide 64N)
G I G A V L K V L T T G L P A L I S W I D-NH_2	(Peptide 64D)
G I G A V L K V L T T G L P A L I S W I H-NH_2	(Peptide 64H)
G I G A V L K V L T T G L P A L I S W I I-NH_2	(Peptide 64I)
G I G A V L K V L T T G L P A L I S W I F-NH_2	(Peptide 64F)
G I G A V L K V L T T G L P A L I S W I S-NH_2	(Peptide 64S)
G I G A V L K V L T T G L P A L I S W I S-NH_2	(Peptide 64M)

The major finding of the present study described in the Examples was that the disulphide dimer, peptide 01B, had a lytic activity approaching that of melittin (on a molar basis), despite the reports that shortened sequences of melittin lose activity, reaching zero on the loss of the basic C-terminal sequence. This was further demonstrated by alkylating peptide 01B (peptide 01D) to give a non-active monomeric peptide. However, if the cysteine thiol was blocked by acetamidomethyl (ACM), a non-charged group, lytic activity was retained, only marginally reduced compared with melittin. These results suggest that the presence of the negative charge on the carboxymethyl group could be responsible for the loss of activity and that lytic activity may not be dependent on the formation of a dimer. If the position of the cross-link is placed at the N-terminal, instead of the C-terminal, the activity is reduced but is still significant (peptide 29).

The sequence requirements of these dimers appears different to that of melittin, and the peptide analogues described earlier, as a dimer (peptide 41) based on the sequence of one of these analogues, peptide 34, has no significant activity. Various substitutions indicate that proline 14 and a basic side chain at position 7 are essential for haemolytic activity of the dimeric peptides. Furthermore acetylation of any of these dimeric peptides reduces or destroys their activity (peptides 01C, 42B, 44B, 46B and 50A). We have included an aromatic residue at position 19 in all of these peptides because of the reported important role tryptophan plays in the activity of melittin.

We thus have the situation where it has been demonstrated that lysine 7 is not important for the lytic activity of melittin and melittin-like peptides, but either lysine or arginine appears essential for the activity of the dimeric peptides. This is reinforced by the observation that acetylation of peptide 01B (peptide 01C) destroys its activity, whereas acetylation of melittin has no effect (Habermann & Kowallek (1970) Hoppe - Seyler's Z. Physiol. Chem. 351 : 884-890).

A similar observation is evident with proline 14, where its inclusion enhances activity of the dimer, but removal of proline from melittin has either no effect or even a positive enhancement of activity, peptides 10 and 34 (Degrado et al (1983) Biophys. J. 37: 329-338; Werkmeister et al (1993) Biochim. Biophys. Acta 1157:50-54.

A summary of these results would suggest that a lytic peptide with activity approaching that of melittin can be produced from an approximate 20 residue amphipathic peptide providing the N-terminal amino group remains free and the peptide includes a lysine or arginine residue at position 7, a proline at 14 and probably an aromatic residue at 19. A cross link at the N-terminal is less effective than one at the C-terminal. Despite these

conclusions it is noted that peptide O1B is the most lytic of the dimeric peptides produced, and that there is clearly some other sequence parameter involved, other than those already discussed. Nonetheless, this study provides significant information for the generation of a unique class of toxic peptides from truncated analogues by selection of certain essential residues and dimerisation.

Example 7 Preparation of Recombinent Immunotoxins

Figure 9 depicts cartoon diagrams of immunotoxins.

Figure 10 describes specific oligonucleotides which were synthesised and used to construct an scFv (as shown in Figure 9a) as an expression cassette in the NcoI-EcoR1 site of vector pGC. (Coia et al, J. Immunol Methods, 1996 192 13-23). The synthesised protein, after expression and signal peptide cleavage, comprised N-terminal FLAG octapeptide fused to the tripeptide AVY (PSA cleavage site) fused to DERO1B cytolytic peptide fused to GGGG spacer fused to an scFv. In this example the scFv was derived from hybridoma 1C3 (glycophorin specific; Coia *et al*, 1996). The recombinant immunotoxin was purified (cytolytically inactive) until presentation *in vivo* where activation is dependant on cleavage by prostate-specific antigen (PSA). It should be recognised that any alternative enzymatic cleavage sequence could replace AVY, and that any cytolytic peptide described in this specification could replace DER01B and that any scFv can replace 1C3.

Figure 9a depicts an immunotoxin in which an inactivating leader peptide is joined by a proteolytic cleavage sequence (X) to a cytolytic peptide joined to an scFv (in this case in the orientation VH-VL). The details of the genetic constructs are described in Figure 10.

Figure 9b depicts a chemically or genetically conjugated Fab comprising one heavy chain fused to a

cytolytic peptid and a second light chain molecule fused to another proteolytically sensitive linker sequence fused to another cytolytic peptide. The details of methods of genetic construction will be known to persons skilled in the art from previous descriptions such as Dolezal *et al*, (Immunotechnology, 1995, 1 197-219). It should be recognised that the cytolytic peptides will be disuphide bonded through cysteine residues and thereby remain inactive until proteolytic cleavage occurs at the linker regions.

Figures 9c and d depict reverse orientation forms of Figures 9a and 9b.

Applications of the Invention

These peptides can be used in a wide range of applications which exploit the property that the peptides have little or no biological activity either in the monomeric state and/or when the C-terminal residue is negatively charged, but are highly lytic on dimerisation and/or blockage of the negative charge.

1. Biosensors

In this application it is envisaged that one lytically inactive monomeric form of the peptide is attached to an antigen and a second is attached or adsorbed to a solid support or to a membrane, a receptor, a bound antibody or a Fab fragment, the measurement being as a result of membrane lysis or by ion-channel formation. In the latter case the dimeric form of the molecule constitutes a voltage-gated ion channel, so that dimerisation results in current flow, effectively providing a redox switch if dimerisation is via a disulphide bond.

2. Immunotoxins

In this application th peptide is attached, by means of a suitable cross-linking agent, either as a monomer or as a dimer, to an antibody or Fab fragment, Fv fragment or ScFv fragment or other antigen-specific fragment thereof, which is specific for a target cell. Preferably the antibody is directed to a target antigen which does not mediate internalization, more preferably a carcinoma-specific antigen. The synthetic peptides described herein are designed for ease of linking to antibodies or fragments thereof. It is contemplated that they will be useful as replacements for toxic peptides such as Ricin A chain. Representative constructs are depicted in Figure 9.

In an alternative approach the peptide of the invention is expressed either as a monomer or as a heterodimer by recombinant DNA technology, together with the Fab, Fv or ScFv fragment of an antibody. The immunotoxin can be utilised as the monomer, or can be dimerised prior to utilisation.

In another alternative, the monomeric peptide is crosslinked to the immunoglobulin or Fab fragment, with the same or different monomer attached thereto. One monomer chain is attached to the protein and the second free peptide crosslinked to it, preferably via the formation of a disulphide.

A third alternative is to couple a peptide of the invention to a growth factor, such as epidermal growth factor or transforming growth factor-α, either chemically or as a fusion protein, which would enable the peptide of the invention to target cells bearing receptors for these growth factors.

The peptides of the invention can overcome the problem of a cytolytic molecule killing the cell in which it is produced, by expressing half of the dimer, and then for example oxidising this at the target site to form the

active molecule. Other methods will readily occur to the person skilled in the art.

For targeting tumour cells, such as prostate cancer cells, a peptide of the invention can be expressed with one or more tyrosine residues incorporated directly adjacent to or near to the peptide sequence, which are then cleaved by the target cell. While such cleavage may not be essential for activity, activity could be increased in this way. We have found that prostate-specific antigen has protease activity which induces specific cleavage at tyr-tyr (see PCT/AU95/00536 entitled "Assay for the Detection of proteases").

3. Bactericides/antibiotics

Using two different designed sequences for each helical segment it is possible to produce a dimer which will lyse bacterial cells but not mammalian cells. For example the amphipathic helical sequence:

H-G I G A V L K V L T T G L P A L I S W I C $-NH_2$

may be coupled to a suitable segment from a class of peptides known as cecropins. Some cytolytically- and antibacterially-active cecropin A - melittin hybrid peptides are known (Diazachirica *et al*, Eur. J. Biochem., 1994 224 257-263; Fernandez *et al*, Biopolymers, 1994 34 1251-1258). Through the formation of a disulphide, such a segment may suitably be:

K W K L F K K I E K V G Q C $-NH_2$.

Alternatively, to aid in the formation of the disulphide, the cysteine residue may be replaced by a penicillamine residue in one of these peptides.

These peptide sequences are only examples, and potentially any amphipathic helical sequence could be

substituted for the first peptide and likewise several alternative sequences derived from the cecropins could be substituted for the second peptide.

4. **Therapeutic Treatment of Disease**

The peptides of the second embodiment of the invention are thought to be particularly useful in the treatment of diseases where it is necessary to selectively destroy cells such as in cancer therapy or in other therapeutic situations where it is desirable to destroy cells, preferably selectively. These peptides can be used in applications which exploit the property that the peptides have little or no biological activity until activated by an appropriate enzyme.

In cancer treatment the peptide is designed to be released from its inactive form in the presence of a tumour specific protease, such as PSA. In an alternative approach an appropriate enzyme can be delivered to the site of a tumour by an antibody prior to treatment with the peptide.

In a second alternative the pre-toxin is attached, by means of a suitable cross-linking agent, either as a monomer or as a dimer to an antibody or Fab fragment. Alternatively the monomeric peptide is expressed with the Fab fragment.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

CLAIMS

1. A peptide with lytic activity, having an amphipathic α-helix of sufficient length and character to allow the peptide to function lytically, wherein the N-terminal and/or C-terminal of said peptide comprises one or more moieties which result in an increased positive charge compared to the charge of a peptide of identical amino acid sequence and structure but not comprising said moiety.

2. A peptide according to Claim 1 which the amphiphathic α-helix is derived from a naturally-occurring cytopathic amphipathic peptide, selected from the group consisting of melittin; other insect venoms; wasp, ant, and scorpion venoms; magainins, bombolitins, and mastoparans; and other known cytopathic amphipathic peptides.

3. A peptide according to Claim 1 comprising two α-helices which are the same.

4. A peptide according to Claim 1 comprising two α-helices which are different.

5. A peptide according to any one of Claims 1 to 4 comprising up to 21 amino acids, in which the α-helix has at least 45% amino acid homology to melittin.

6. A peptide according to Claim 1, having hydrophilic amino acids at positions 3, 4, 7, 10, 11, 14 and 18, of which at least two are lysine and/or arginine; and hydrophobic amino acids at positions 1, 2, 5, 6, 8, 9, 12, 13, 15, 16, 17, 19 and 20.

7. A peptide according to Claim 6 in which the amino acid at position 7 is lysine or arginine.

8. A peptide according to Claim 6 or Claim 7 in which the amino acid at position 14 is proline or hydroxyproline.

9. A non-naturally occurring peptide with lytic activity, having an amphipathic α-helix of sufficient length and character to allow the peptide to function lytically, wherein the C-terminal residue of said peptide is not negatively charged and wherein absence of a negative

charge at the C-terminal residue is provided by blocking the n gative charge of said r sidue.

10. A peptide according to Claim 9 in which a cysteine residue is present at either the N-terminal or the C-terminal end of the α-helix.

11. A peptide according to Claim 10 in which the C-terminal residue is a cysteine residue which has been neutralised.

12. A peptide according to Claim 1, Claim 3 or Claim 4, comprising two amphipathic α-helices which are not themselves lytic, linked to form a dimer which has lytic activity.

13. A peptide according to Claim 12 in which the components of the dimer are linked via a desulphide bond.

14. A peptide according to Claim 13 in which the disulphide bond is effected by having a cysteine or cysteine-amide residue at the C-terminal of each of the components of the dimer, or via a bridge moiety such as $-CH_2S(CH_2)_nSCH_2-$, where n is 1 to 4.

15. A peptide according to any one of claims 12 to 14 in which the amphiphathic α-helix is derived from a naturally-occurring cytopathic amphipathic peptide, selected from the group consisting of melittin; other insect venoms; wasp, ant, and scorpion venoms; magainins, bombolitins, and mastoparans; and other known cytopathic amphipathic peptides.

16. A peptide according to Claim 12 which is a cytotoxic dimer of an amphipathic peptide, in which the amphipathic peptide has the general formula:

Ω Ω X X Ω Ω X Ω Ω X X Ω Ω (X, P or hypro) Ω Ω Ω X Ω Ω (C or Z) or (C or Z) Ω Ω X X Ω Ω X Ω Ω X X Ω Ω P Ω Ω Ω X Ω Ω;

and wherein the symbols have the following meanings:

Ω = glycine; a hydrophobic amino acid including but not limited to alanine, isoleucine, leucine, valin , and norleucine; or ϕ;

Σ = asparagin , glutamin , serine or threonine;
Φ = phenylanine, tryptophan, tyrosine, a non-naturally occurring aromatic amino acid, or a synthetic aromatic residue; and
X represents a sequence of ? hydrophilic amino acids, at least one of which is a basic amino acid.

and wherein C or Z may be at either end of the peptide but not at both ends.

17. A peptide according to Claim 17 in which two or more of the hydrophilic amino acids are basic amino acids.

18. A peptide according to Claim 16 or Claim 17 in which the amphipathic peptide has the general formula

Ω Ω Σ Σ Ω Ω (K or R) Ω Ω Σ Σ Ω Ω P Ω Ω Ω Σ Φ Ω (C or Z)
or Ω Ω Ω Ω Ω Ω (K or R) Ω Ω Σ Σ Ω Ω P Ω Ω Ω Σ Φ Ω (C or Z)
or (C or Z) Ω Ω Σ Σ Ω Ω (K or R) Ω Ω Σ Σ Ω Ω P Ω Ω Ω Σ Φ Ω
or Ω Ω Ω Ω Ω Ω (K or R) Ω Ω Σ Σ Ω Ω P Ω Ω Ω Σ Φ Ω

and wherein the symbols have the following meanings:

R = is arginine
K = lysine
hypro = hydroxyproline
P = proline
C = cysteine
Z = is an amino acid which is not negatively charged, or is either a cross-linking agent, or a blocking group.

19. A peptide according to Claim 9 selected from the group consisting of

G I G A V L K V L T T G L P A L I S W I C -NH_2 (Peptide 01B)
C G I G A V K L V L T T G L P A L I S W I -NH_2 (Peptide 29)
2(G I G A V L K V L T T G L P A L I S W)K -NH_2 (Peptide 30)
X L Q S L V S L V Q S L V S L V L W F L R S R K N N -NH_2 (Peptide 34)
L L Q S L V K L V Q S L V P L V L Q F L C -NH_2 (Peptide 42)
L L Q S L V K L V Q S L V S L V L Q F L C -NH_2 (Peptide 44)
G L G A L V K L V T S G V P L V L S W L C -NH_2 (Peptide 56)

A V R G I G A V L K V L T T L P A L I S W I C -NH_2 (Peptide 50)
Ac A V R G I G A V L K V L T T G L P A L I S W I C -NH_2 (Peptide 50A)
G I G A V L K V L T T G L P A L I S W I M-NH_2 (Peptide 64M)
S S L G I K A V L K V L T T G L P A L I S W I A -NH_2 (Peptide AP11)
S S L G I G A V L K V L T T G L P A L I S W I A -NH_2 (Peptide AP13)

where -NH_2 indicates the C-terminal amide group; in which the peptide may optionally be amidated, and/or acylated at the N terminal.

20. A peptide according to Claim 19, in which the helical dimer is

G I G A V L K V L T T G L P A L I S W I C
|
G I G A V L K V L T T G L P A L I S W I C

21. A peptide which is capable of lytic activity, comprising an amphipathic α-helix of sufficient length and character to allow the peptide to function lytically, and

(a) a cleavable moiety located amino terminal to said α-helix of sufficient length and character to allow the peptide to function lytically, and

(b) a negatively-charged carboxy terminal residue of said peptide, wherein the negative charge prevents or retards lytic function of said peptide and wherein removal of said charge by blocking with a suitable group results in lytic activity.

22. A peptide according to Claim 21, in which the cleavable moiety is pre-sequence Acetyl-AVY-, Acetyl-SSGYSNT- or Acetyl-AVE-.

23. A peptide according to Claim 21 or Claim 22 in which the cleavable sequence is selectively removable by enzymatic means.

24. A composition comprising a peptide according to any one of Claims 1 to 23, together with a pharmaceutically-acceptable carrier.

25. A p ptide according to any one of claims 1 to 23

linked to a carrier molecule.

26. A carrier-linked peptide according to Claim 25 in which the carrier molecule is a targeting agent.

27. A carrier-linked peptide according to Claim 25 or Claim 26 in which the carrier molecule is an antibody, an antigen-specific fragment or analogue of an antibody, epidermal growth factor or transforming growth factor-α.

28. A carrier-linked peptide according to claim 27 in which the antibody, or fragment or analogue thereof, is specific for a cell-surface antigen.

29. A carrier-linked peptide according to Claim 28 in which the antigen is a cancer-specific antigen.

30. A carrier-linked peptide according to claim 29 in which the cancer is a solid tumour.

31. A composition comprising a carrier-linked peptide according to any one of claims 25 to 29, together with a pharmaceutically-acceptable carrier.

32. A method of modulating the lytic activity of a peptide, said peptide comprising an amphipathic α-helix of sufficient length and character to allow the peptide to function lytically, and optionally comprising additional amino acids, said method comprising, under suitable conditions,

(a) increasing the positive charge of said peptide by adding to the N and/or C terminal one or more moieties which increase the positive charge of the peptide, or

(b) decreasing the positive charge of said peptide by removing from the N and/or C terminal one or more moieties which decrease the positive charge of the peptide

wherein step (a) results in an increase of lytic activity or activation of lytic activity and step (b) results in a decrease or inactivation of lytic activity.

33. A method of activating a lytically inactive peptide, wherein said inactive peptide comprises an

amphipathic α-helix of sufficient length and character to allow the peptide to function lytically and

a) a cleavable moiety located amino terminal to said α-helix, wherein said moiety inactivates lytic function of said peptide and wherein removal of said moiety results in a lytically active peptide, and/or

b) a negatively charged carboxy terminal residue wherein the negative charge may be removed by blocking with a suitable group which results in lytic activity

said method comprising, under suitable conditions, treating said peptide with

c) a cleaving agent to remove said moiety and/or

d) a blocking agent to remove the negative charge of said residue such that said peptide becomes lytically active.

34. A method of inactivating a lytically active peptide wherein said peptide comprises an amphipathic α-helix of sufficient length and character to allow the peptide to function lytically, and/or a non-negatively charged carboxy terminal residue, and where said peptide is optionally in the form of a dimer comprising two α-helices, said method comprising, under suitable conditions,

a) linking a cleavable moiety amino terminal to said α-helix and/or

b) creating a negative charge on said carboxy terminus.

35. A method according to claim 31 in which the cleavable moiety is an amino acid presequence which reduces the overall positive charge of the peptide.

36. A composition according to Claim 24 or Claim 30, further comprising one or more antibiotics, toxic anions, anti-tumour agents, antibacterial agents, antiviral agents or antiparasitic agents.

37. A method of treatment or alleviation of cancer or of a manifestation of infection with human immunodeficiency virus, comprising th st p of administering an effectiv

amount of a peptide according to any one of Claims 1 to 23, either alone or coupled to a carrier molecule, to a subject in need of such treatment.

38. A method of treatment of a microbial infection, comprising the step of administering an effective amount of a peptide according to any one of Claims 1 to 23 to a subject in need of such treatment, said dimer comprising an amphipathic helical sequence of the invention and a cecropin or a cecropin-like peptide.

39. A biosensor, comprising a first monomeric form of a peptide according to any one of Claims 1 to 23 linked to an antigen and a second monomeric form of a peptide of the invention linked or adsorbed to a solid support or to a membrane, a receptor, or a bound antibody or Fab, Fv, scFv or other antigen-binding fragment or analogue thereof, such that an analyte to be detected induces dimerisation of the monomers.

40. A peptide, carrier-linked peptide, composition, method or biosensor, substantially as hereinbefore described with reference to the examples and drawings.

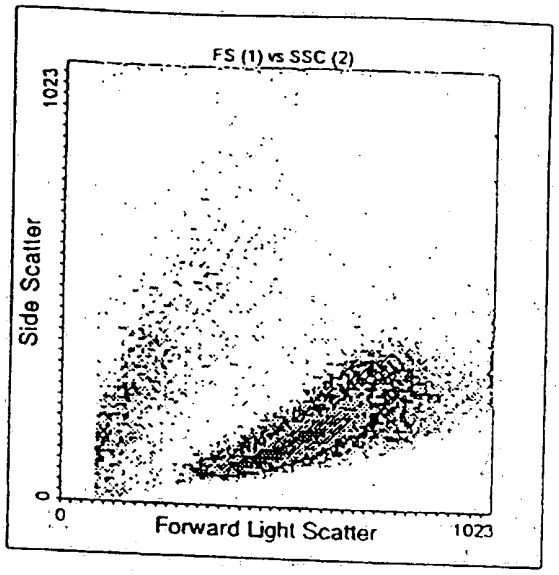


Figure 1a

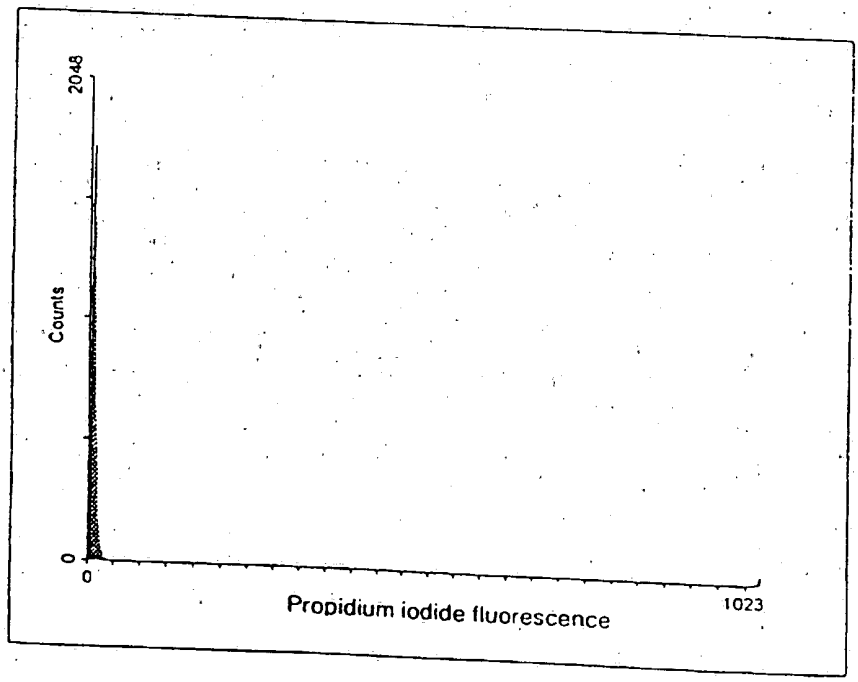


Figure 1b

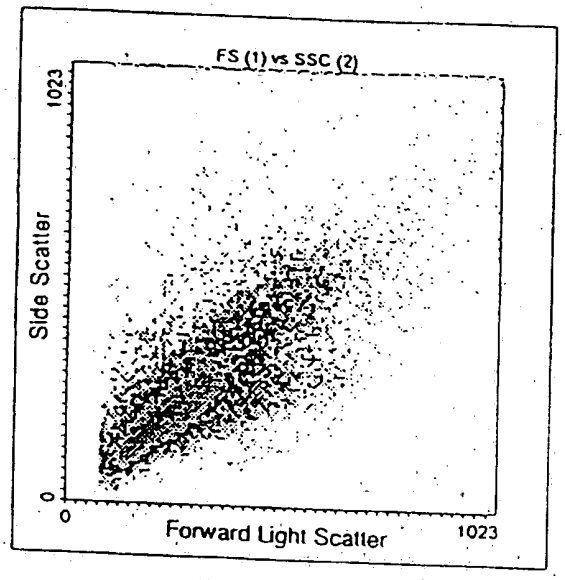


Figure 2a

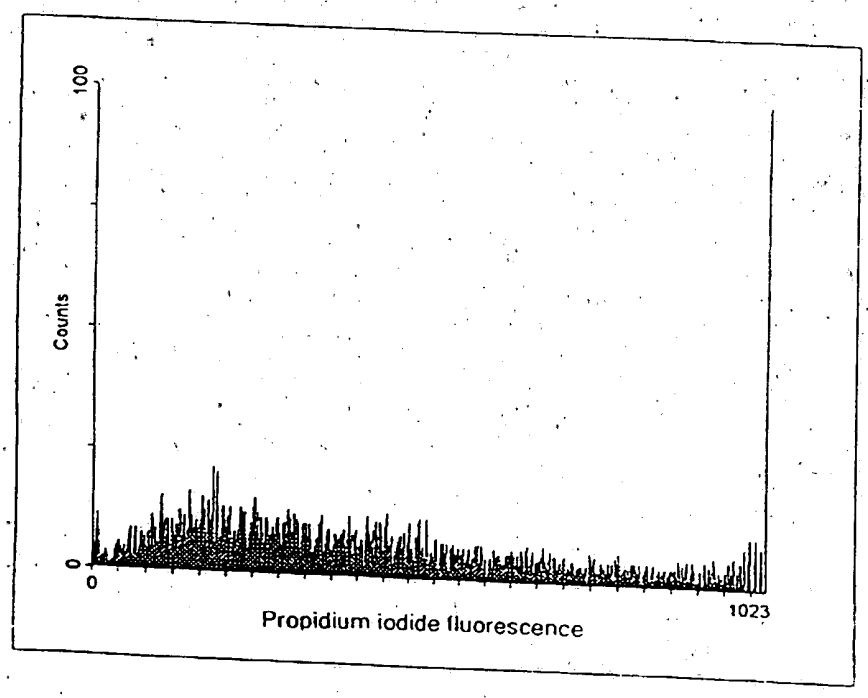


Figure 2b

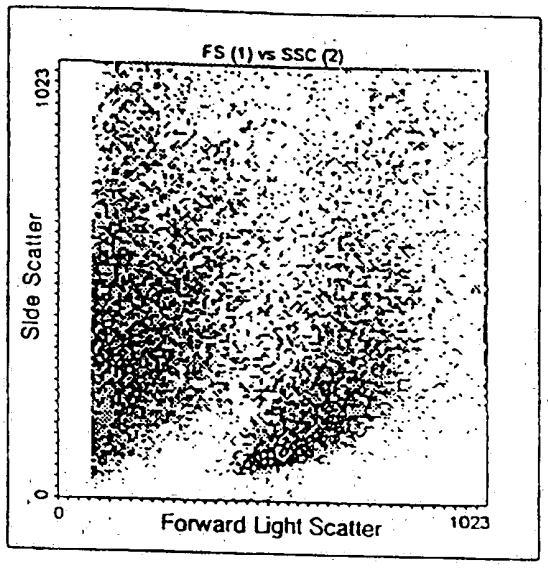


Figure 3a

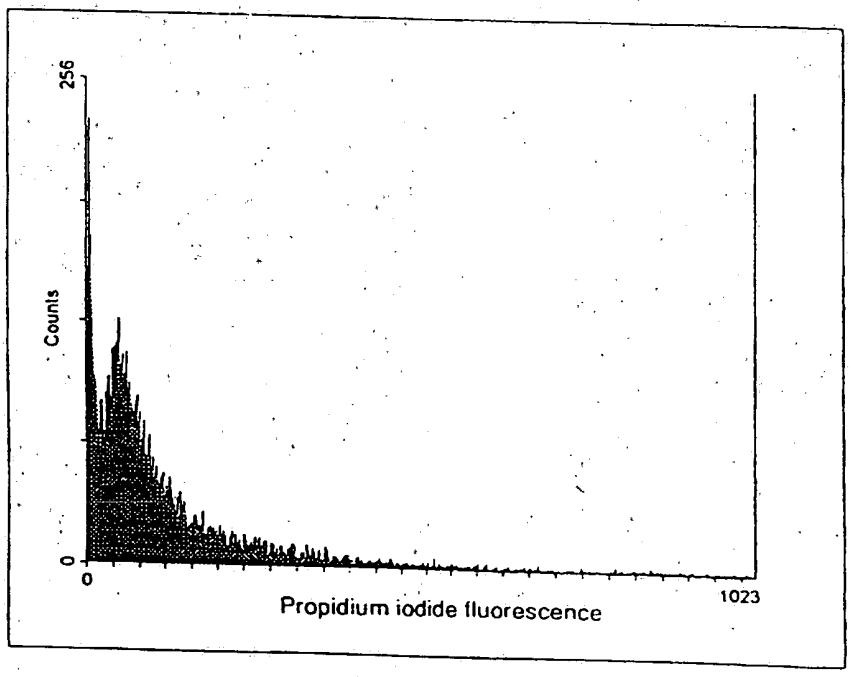


Figure 3b

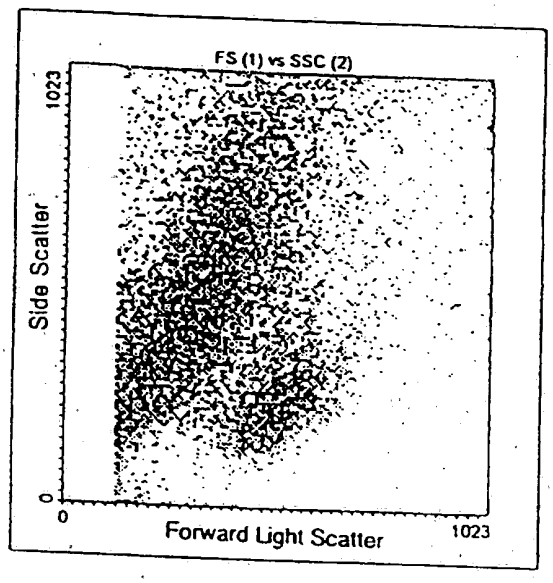


Figure 4a

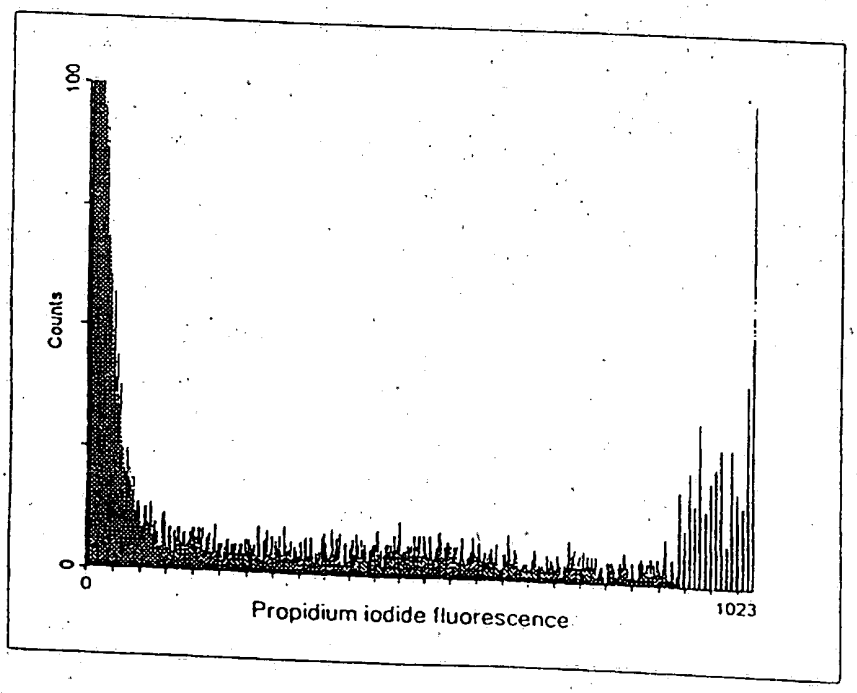


Figure 4b

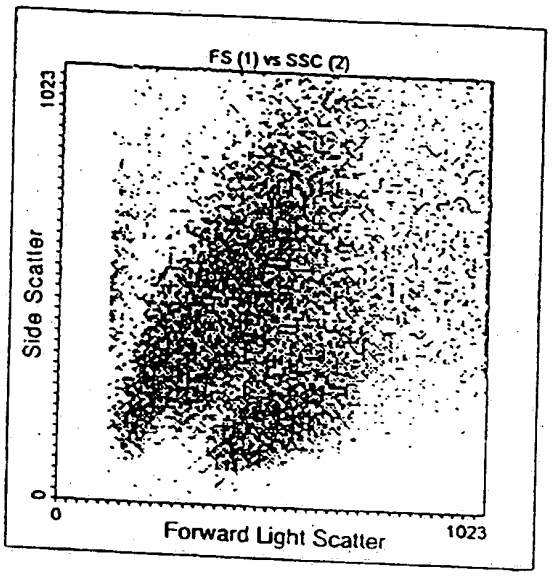


Figure 5a

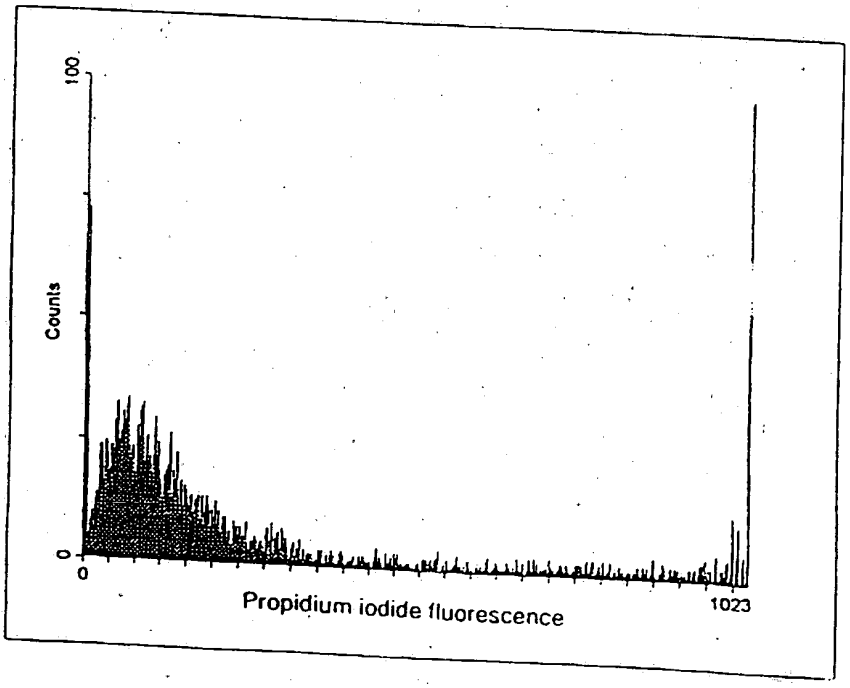


Figure 5b

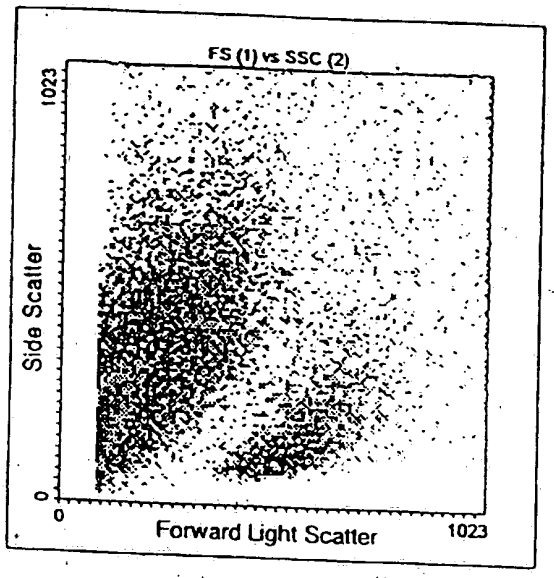


Figure 6a

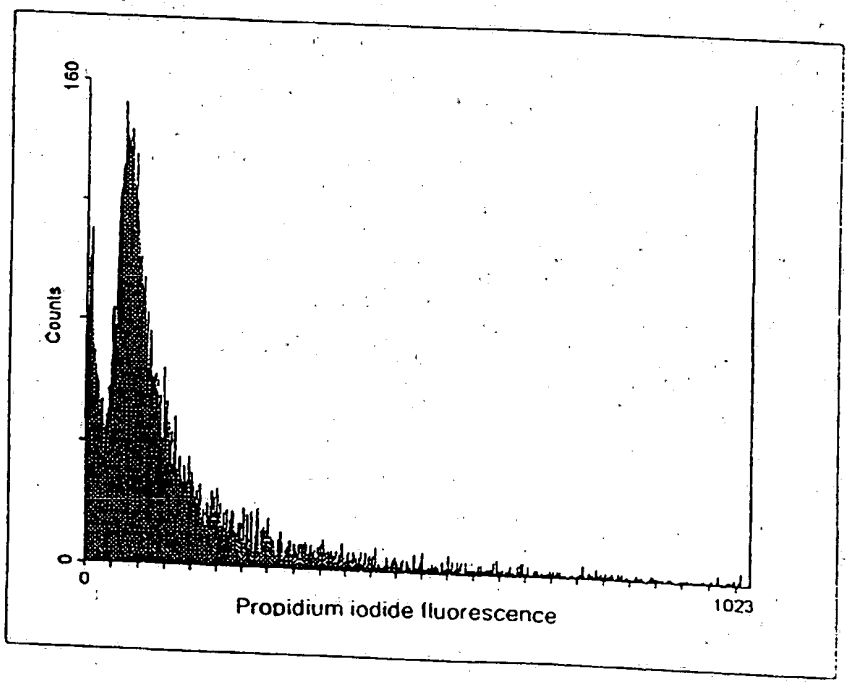


Figure 6b

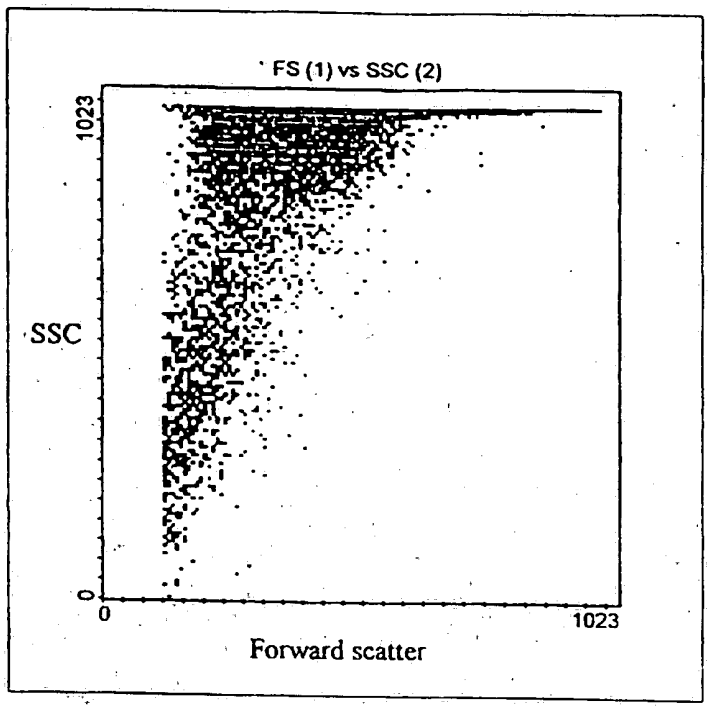


Figure 7a

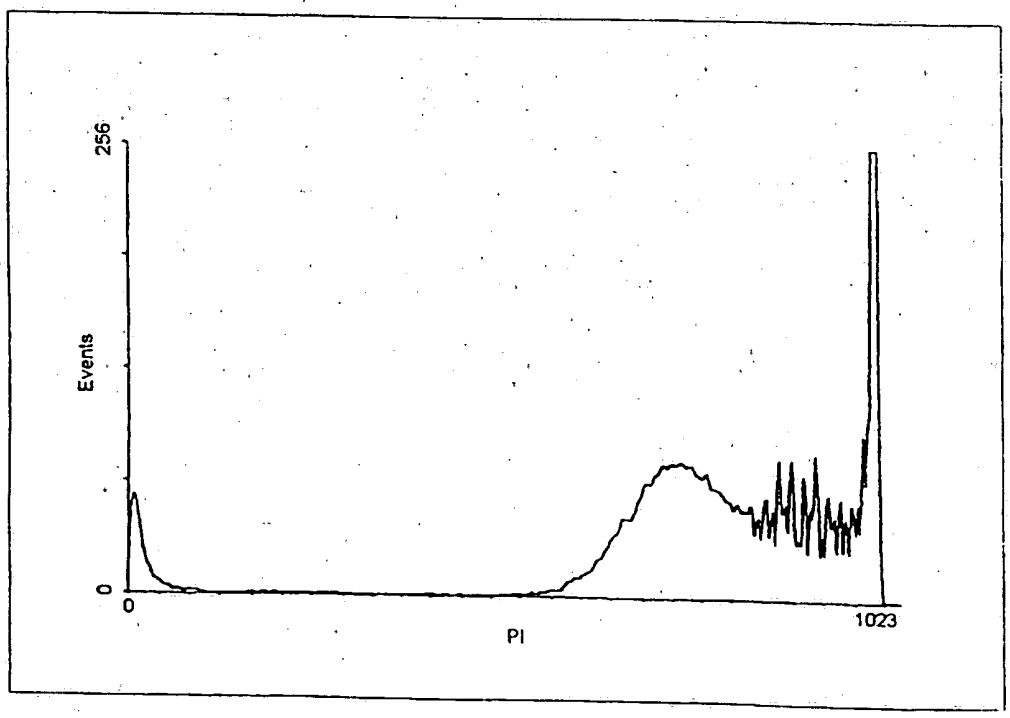


Figure 7b

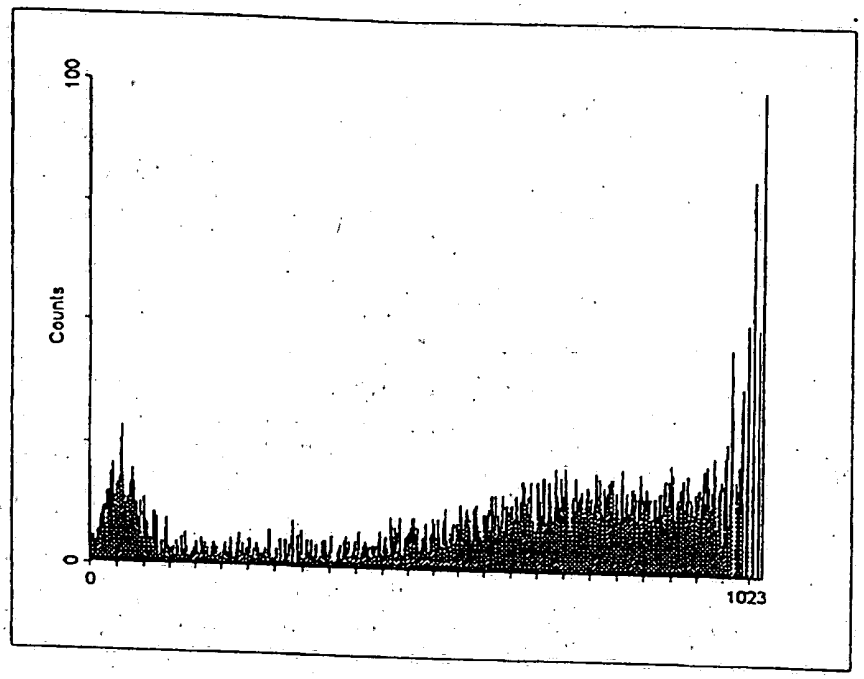


Figure 8a

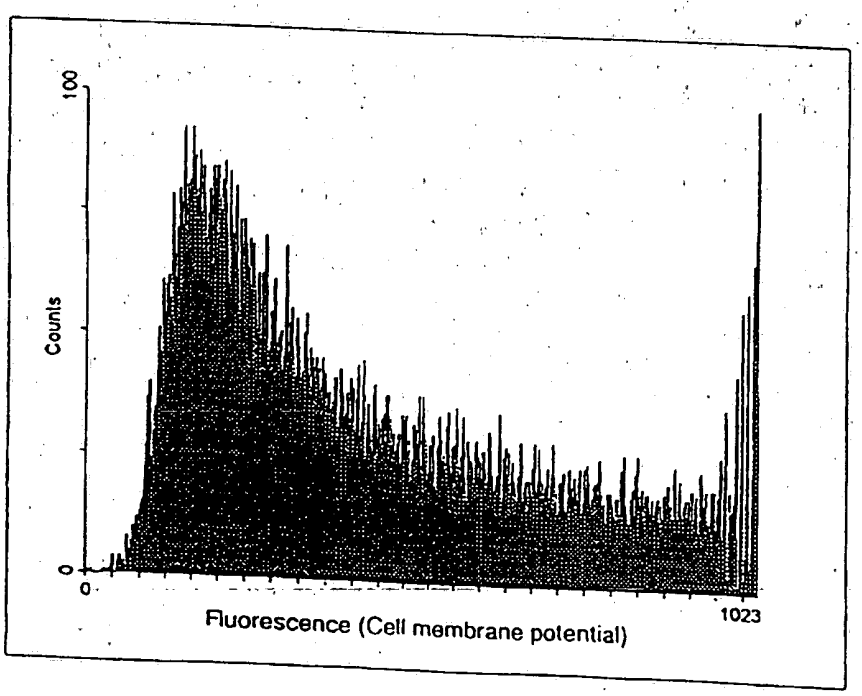


Figure 8b

Immunotoxin Examples

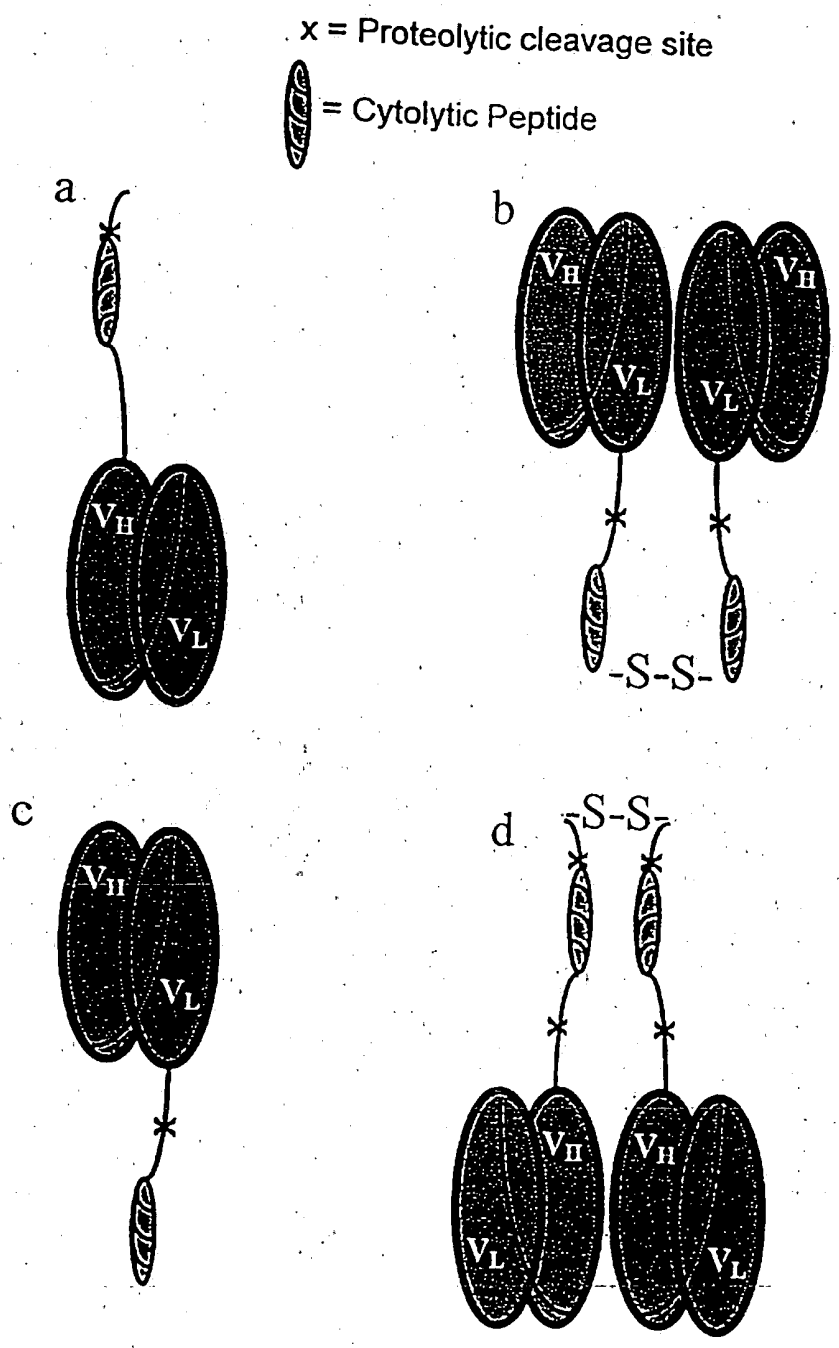
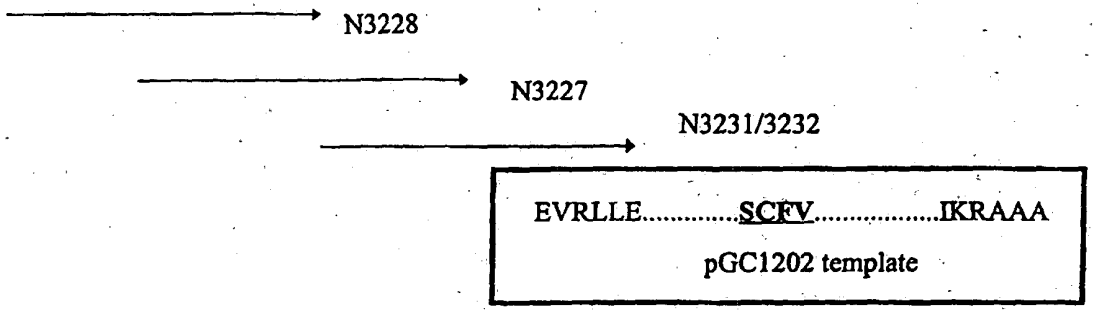


Figure 9

PCR cloning stategy to create :
" NcoI-FLAG-AVY-DER01B-spacer-Scfv-EEF/Btag-stop-EcoR1" cassette to drop into pGC1202 (NcoI/EcoR1 cut).



A M A D Y K D D D D K A V Y G I G A V L K V L

N 3228 1. aaa aaa GCC ATG GCC GAT TAT AAA GAT GAT GAT GAT AAA GCG GTG TAT GGC ATC GGC GCG GTG→

Nco 1

N3227 2. GGC ATC GGC GCG GTG CTG AAA GTG CTG

T T G L P A L I S W I C G G G G E V R L L E

ACC ACC GGC CTG CCG GCG CTG ATC AGC TGG ATC TGC→

N3231 3. CTG ATC AGC TGG ATC TGC GGT GGT GGT GGT GAG GTG AGG CTT CTT GAG→

* F E E A A A R K I

N3226 4.← ttt ttt GAA TTC TTA AAA TTC TTC TGC GGC CGC CCG TTT GAT

EcoR1

Figure 10

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU97/00160

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07K 7/08, 14/31, 14/35, 17/00; G01N 27/327; G01N 33/68; A61K 38/12, 38/16, 38/17.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
see electronic database box

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
see electronic database box

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
(see attached sheet)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biochemistry (1991), vol. 30 pages 4671-4678. S.E.Blondelle and R.A. Houghton: "Hemolytic and Anti-Microbial activities of twenty four individual omission Analogues of melittin" (see in particular, page 4673, column 1 last paragraph and page 4676, column 1, second paragraph).	12,32

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
17 April 1997

Date of mailing of the international search report
23 APR 1997

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU97/00160

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,96/18104, Torrey Pines Institute for Molecular Studies (published 13 June 1996)	1-40
A	Biophysical Journal, volume 37, no.1, pages 329-338 (January 1982). W.F. de Grado et al, "Kinetics and mechanism of hemolysis induced by melittin and by a synthetic melittin analogue".	1-40

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU97/00160

Box (continued from box B)

electronic data base (cont.):

WPAT:USPM:JAPIO:keywords:
melittin or magainin or cecropin or bombolitin or mastoparan or haemolysin or hemolysin or amphipathi:( ) peptide and lytic or lysis or h(a)emolysis or h(a)emolytic

medline: keywords:
melittin analog/ct

chemical abstracts: keywords:
melittin/cn and lytic or lysis or hemoly? or haemol?

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU97/00160

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO	96/18104	AU	33714/95
			END OF ANNEX